

Response of rainbow trout transcriptome to model chemical contaminants

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Abstract

We used high-density cDNA microarray in studies of responses of rainbow trout fry at sublethal ranges of β -naphthoflavone, cadmium, carbon tetrachloride, and pyrene. The differentially expressed genes were grouped by the functional categories of Gene Ontology. Significantly different response to the studied compounds was shown by a number of classes, such as cell cycle, apoptosis, signal transduction, oxidative stress, subcellular and extracellular structures, protein biosynthesis, and modification. Cluster analysis separated responses to the contaminants at low and medium doses, whereas at high levels the adaptive reactions were masked with general unspecific response to toxicity. We found enhanced expression of many mitochondrial proteins as well as genes involved in metabolism of metal ions and protein biosynthesis. In parallel, genes related to stress and immune response, signal transduction, and nucleotide metabolism were down-regulated. We performed computer-assisted analyses of Medline abstracts retrieved for each compound, which helped us to indicate the expected and novel findings.

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Multiple gene expression profiling is increasingly used in toxicology for development of drugs and in their risk assessment, finding of diagnostic markers and in mechanistic studies of toxicity, classification of toxic compounds and development of reference knowledge bases [1–4]. Merge of high-throughput analytic technologies with bioinformatics and data mining is referred to as a novel scientific discipline, toxicogenomics. Methods of functional genomics are rapidly expanding towards new species, which are important for ecotoxicology and environmental monitoring.

Development of microarray for studies of the effects of pollutants on marine teleost fish plaice (*Platichthys flesus*) was reported recently [5]. We constructed a high-density cDNA microarray with more than 1300 genes

for salmonid fish rainbow trout (*Oncorhynchus mykiss*). Experiments with model contaminants were designed to assess the potential of this platform for environmental research. Yolk-sac fry were exposed short term to sublethal range of four aquatic toxicants, representing a ligand acting through the aryl hydrocarbon receptor (AhR), a non-essential heavy metal, an industrial chlorinated solvent, and an environmentally relevant polycyclic aromatic hydrocarbon (PAH). β -Naphthoflavone was used as a surrogate of model chemical 2,3,7,8-tetrachlorodibenzo-*p*-dioxin [6] due to occupational reasons. The transcriptomic response was analyzed in whole body. Overall, our study was focused on discrimination of effects of different chemicals, selection of potential diagnostic biomarkers, and understanding of molecular mechanisms of toxicity. As microarray analyses commonly reveal a plethora of differentially expressed genes, the interpretation of results requires

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tedious search through public databases and scientific literature. In the present work this task was facilitated by combination of functional annotations with statistical inference and development of a tool for computer-assisted analysis of Medline abstracts.

Materials and methods

Experiments with fish. Recently hatched fry of rainbow trout were acclimated at 10.5 °C for at least 10 days before experiments. Exposure concentrations were set to the sublethal area relative to the acute lethality for freshwater teleost fishes [7]. Because abiotic factors as well as the animal species and its stage of life cycle affect the lethal concentration (LC) to a large extent, approximate acute lethal levels of waterborne β -naphthoflavone, cadmium, carbon tetrachloride, and pyrene were determined or assured by preliminary experiments with small numbers of animals ($n = 3$ per concentration). In 4 days no apparent mortality revealed for β -naphthoflavone, carbon tetrachloride, and pyrene at 200, 40, and 100 $\mu\text{g/L}$, respectively, the highest concentrations of final exposures. LC-value (96 h) for cadmium was estimated to be between 0.2 and 0.7 mg/L. The sublethal range was set to be between 20% and 75% of the expected LC. Three dose levels—termed low, medium, and high—were conducted. Fry were exposed to β -naphthoflavone, carbon tetrachloride, pyrene and the high dose of Cd (0.5 mg/L) for 4 days. Because increasing mortality was observed at exposures to cadmium, we carried out one more experiment with lower doses (0.05 and 0.25 mg/L) for 24 h, these being referred to as low and medium. Three replicate experiments were made for every exposure concentration, with 9 or 10 fry in 3 cm water depth. In order to separate possible effect of vehicle (β -naphthoflavone and pyrene), both clean and DMSO controls were carried out. Strict control of water temperature within ± 0.1 °C was ensured. Fish were snap-frozen and preserved in liquid nitrogen.

Design of microarray. For preparation of glass cDNA microarray we used subtracted EST libraries and selected clones from a single normalized cDNA library [8]. The newly identified cDNA sequences were compared with nucleotide databases using stand-alone blast [9] and functional annotation was made by the categories of Gene Ontology [10]. The cDNA inserts of 1380 non-redundant clones were amplified with PCR using universal primers and purified with Millipore Montage Plasmid Miniprep-96 Kit. DNA was spotted onto

poly-(L)lysine-coated slides and each clone was printed in six replicates.

Microarray analyses. Total RNA was extracted from whole fish using Trizol reagent (Invitrogen) and four individuals were randomly pooled in each sample. Labeling with Cy3- and Cy5-dCTP (Amersham-Pharmacia) was made using SuperScript III reverse transcriptase (Invitrogen) and oligo(dT) primer; cDNA was purified with Microcon YM30 (Millipore). We used a dye swap experimental design [11,12] and each sample was hybridized to two microarrays. For the first slide, test and control cDNA were labeled with Cy5 and Cy3, respectively, and for the second array dye assignment was reversed. The slides were pretreated with 1% BSA, fraction V, $5\times$ SSC, and 0.1% SDS (30 min at 50 °C), washed with $2\times$ SSC (3 min) and $0.2\times$ SSC (3 min), and hybridized overnight in cocktail containing $1.3\times$ Denhardt's, $3\times$ SSC 0.3% SDS, 0.67 $\mu\text{g}/\mu\text{L}$ polyadenylate, and 1.4 $\mu\text{g}/\mu\text{L}$ yeast tRNA. All chemicals were from Sigma-Aldrich. Scanning was performed with ScanArray 5000 and images were processed with QuantArray (GSI Luminomics). In total, 24 slides were used in this study.

Data analyses. The measurements in spots were filtered by criteria $I/B \geq 3$ and $(I - B)/(S_I + S_B) \geq 0.6$ where I and B are the mean signal and background intensities and S_I, S_B are the standard deviations. After subtraction of mean background, lowess normalization [13] was performed. To assess differential expression of genes, the normalized logintensity ratios were analyzed for every pair of dye-swap slides with Student's t test ($p < 0.01$). The genes were ranked by $\log(P\text{-level})$ and ranks were calculated separately for the up-regulated and down-regulated genes.

Quantitative RT-PCR. Primers (Table 1) were designed to amplify 194–305 b fragments. Synthesis of cDNA with Superscript III reverse transcriptase (Invitrogen) was primed with oligo(dT). Analyses in fish exposed to the high doses of contaminants were carried out using Dynamo SYBR Green kit (Finnzymes) and ABI Prism 7700 (Amersham-Pharmacia).

Text mining. Computer-assisted analysis of Medline abstracts was performed for finding of over-represented scientific terms. Search was made using the names of chemicals as queries and 14,334 abstracts were retrieved in XML-format using NCBI's E-Utilities. The numbers of abstracts including each term were estimated. Two vocabularies were used for search. First, all abstracts were split into separate words and a list of non-redundant terms was composed. The second vocabulary consisted of terms included into Gene Ontology. The Z scores of hypergeometric distribution were determined as

Table 1
Primers used for real-time quantitative RT-PCR

Gene	Model chemical	Name	Sequence (5'–3')
Cytokeratin 16	β -Naphthoflavone	QU1	TTCCAGACCAAGGCAGAGAC
		QL1	CTGGTCACTTGGTTCTGGAG
Thioredoxin	β -Naphthoflavone	QU2	AAATGCATGCCGACGTTCCA
		QL2	CTTCAAGGGGGAGTTCGGTCTA
α -Tubulin	Cadmium	QU3	CAGGTGTCCACGGCTGTGTT
		QL3	GGGAAGTAGATACGGGGGTAGG
Collagenase type IV	Cadmium	QU4	AACATCAGAAACGCCCTCAT
		QL4	TGGTGGTAGTGGTAGTGGAC
Annexin IV	Carbon tetrachloride	QU5	AGTGTGGATGGGGATGTAGG
		QL5	TATTGGGCTGGGGTCTTGAG
GRB2-related adaptor 2	Carbon tetrachloride	QU6	GCCAGAGACCCCAGGAGAT
		QL6	GGCTGAGAGGATGGGGCTGA
Siah2 protein	Pyrene	QU7	GCCTGTTTGAGTGCCTGTCT
		QL7	CTCCTCGTGCTCTGCCTTGT
Programmed cell death 8	Pyrene	QU8	CAACAGCCAAAGTCAAGAGC
		QL8	AAGCCCCAAAGTCAGAGTC

Table 2
Result of computer-assisted analysis of medline abstracts

Abstracts	β -Naphthoflavone, TCDD 5103 abstracts	Cadmium 4317 abstracts	Carbon tetrachloride 1983 abstracts	Pyrene 2931 abstracts
Toxic effect	Allergy	Apoptosis, cytotoxicity, neurotoxicity, shock, stress	Inflammation, necrosis	Cancer, cytotoxicity, DNA-damage, genotoxicity, mutagenesis
Tissues, cells	Adipocytes, B-cells, cardiovascular, endothelium, enterocytes, epidermis, keratinocytes, pituitary, synapse, thymus, thyroid	Bone, brain, endothelium, gut, heart, intestine, kidney, macrophages, muscle, osteoblasts, pancreas, parathyroid, pituitary	Liver	Bone, epithelium, erythrocytes, fibroblasts, lymphocytes
Cellular targets	Nucleus, microsome, proteasome	Chromosome, cytoskeleton, extracellular space, lysosome, membrane, microtubule, mitochondrion, ribosome	Endoplasm, membrane, microsome, mitochondrion	Chromosome, nucleus, spindle
Substances	Hydrocarbon, hydroxysteroid, ions, iron, NAD, NADP	Aminoglycan, glucosamine, glucose, glutathione, magnesium, manganese, peroxide, phosphate, superoxide	Antioxidant, calcium, cholesterol, glucose, glutathione, hydroperoxide, lipid, lipopolysaccharide, lipoprotein, NADPH, peroxide, phospholipid, prostaglandin, sterol, superoxide, triglyceride	Alkyl, NADP, manganese, ribonucleoside
Proteins	Major histocompatibility complex, cathepsin, epoxygenase, carboxykinase, lipoxygenase, immunoglobulin, acetyltransferase, oxidoreductase, keratin, demethylase, monooxygenases, hydroxylase, ras, receptor, cytochrome P450, AhR/ARNT	Amino-peptidase, ATPase, calmodulin, caspase, catalase, dismutase, elastin, glycosylase, GSH, hemoglobin, Hsp70, hydrogenase, jun, lysozyme, peroxidase, phosphatase, tubulin	Aminotransferase, collagenase, cytokine, dismutase, GSH, hydrogenase, lipase, metalloproteinases, nucleotidase, peroxidase, phosphatase, phosphorylase, procollagen	Acetyltransferase, actin, c-Ha-ras, decarboxylase, demethylase, galactosidase, glucuronidase, GST, keratin, monooxygenases, myeloperoxidase, ras, ribosyltransferase, sulfotransferase
Processes, functions	Alkylation, dehydrogenation, gluconeogenesis, glucuronidation, hydroxylation, immunity, methylation, organogenesis, phosphorylation, signaling, transcription	Adhesion, defense, endocytosis, oxidative metabolism, phagocytosis, respiration, transport	Peroxidation	Cytokinesis, metaphase, mitosis, replication

Table includes over-represented terms (exact Fisher's test, $p < 0.05$).

$$p(x|N, K, n) = \frac{\binom{K}{x} \binom{N-K}{n-x}}{\binom{N}{n}},$$

where x and K are occurrence of terms in the test set (abstract retrieved for one compound) and the reference sets (all abstracts); N and K are the numbers of abstracts in the test and reference sets. Enrichment of terms was analyzed with exact Fisher's test ($p < 0.05$).

Results

The studied chemicals are known to be different by toxic effects or modes of action. Computer-assisted analysis of 14,334 Medline abstracts confirmed that β -naphthoflavone, cadmium, carbon tetrachloride, and pyrene act at different tissues, cellular and subcellular targets, and physiological processes and response can be mediated by various proteins, metabolic and regulatory pathways (Table 2). Therefore, besides chemical differences, we could expect also distinct transcriptomic responses. The numbers of differentially expressed genes varied at exposures to the studied chemicals, being equal to 399 in the whole series. The data for these genes were analyzed with hierarchical clustering. The responses to the low and medium doses of all four compounds were well segregated, whereas the highest doses were in a separate clade (Fig. 1).

A number of genes showed stable alterations of expression levels, examples are in Fig. 2. Exposure to β -naphthoflavone induced genes involved in biotransformation of xenobiotics and synaptic transmission (quinone reductase and phenol sulfotransferase, GST) and oxidative stress (peroxiredoxin, thioredoxin-like

protein). Two heat-shock proteins were induced (HSP-90 β and heat-shock cognate 71 kDa), whereas expression of one more chaperone (BAG-family regulator-4) decreased. Most of down-regulated genes encode structural proteins of extracellular matrix (collagen α 1 and 2) and cytoskeleton (cytokeratin 16 and 19, hypothetical protein FLJ20261). Aryl hydrocarbon receptor (AhR), a classical marker of many PCB-congeners and TCDD, was induced at low and medium doses, however, being down-regulated at the highest dose of β -naphthoflavone. Cadmium increased expression of genes involved in remodelling of extracellular matrix (collagenase III and IV, proteoglycan link protein, and connective tissue growth factor), the latter being also activated with β -naphthoflavone. Transcription factor jun-B and CCAAT/enhancer binding protein are known to be activated with heavy metals [14,15]. Similar to the experiment with β -naphthoflavone, exposure to cadmium decreased expression of structural proteins (α -tubulin, cytokeratin 16, and hypothetical protein FLJ20261). Metallothionein was up-regulated only at the highest dose. Exposure to carbon tetrachloride induced genes encoding lipid-binding proteins (annexin IV and V, brain protein 44, and gastrotropin) and enzymes of glycolysis and energy metabolism (GAPDH, enolase, cytochrome c oxidase I, and ubiquinol-cytochrome c reductase). We selected a pair of up-regulated and down-regulated genes for every compound (Table 1) and analyzed their expression with qPCR. Difference between exposed and control fish was significant (Student's t test, $p < 0.05$) (Fig. 3).

To address further molecular mechanisms of toxic effects, we grouped differently expressed genes by functional categories of Gene Ontology and classes represented by only one gene were discarded. We compared

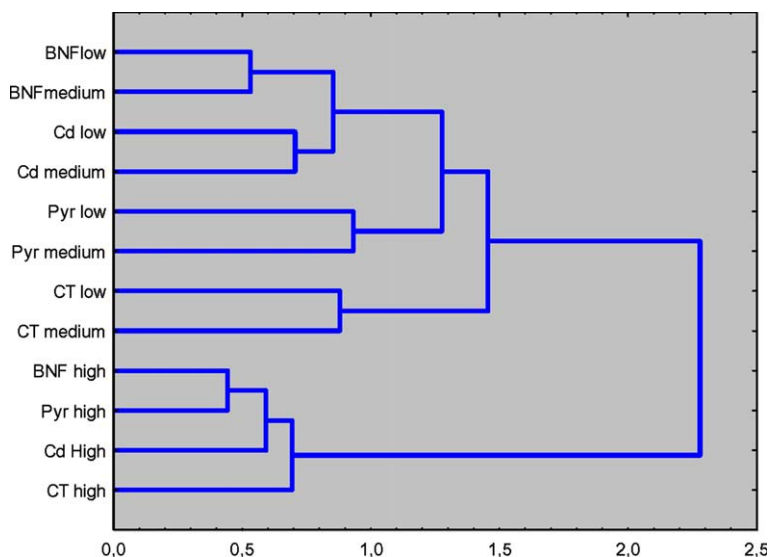


Fig. 1. Response of rainbow trout fry to β -naphthoflavone (BNF), cadmium (Cd), carbon tetrachloride (CT), and pyrene (Pyr) at different levels. The ranks of differentially expressed genes were clustered by Pearson correlations using Ward's method.

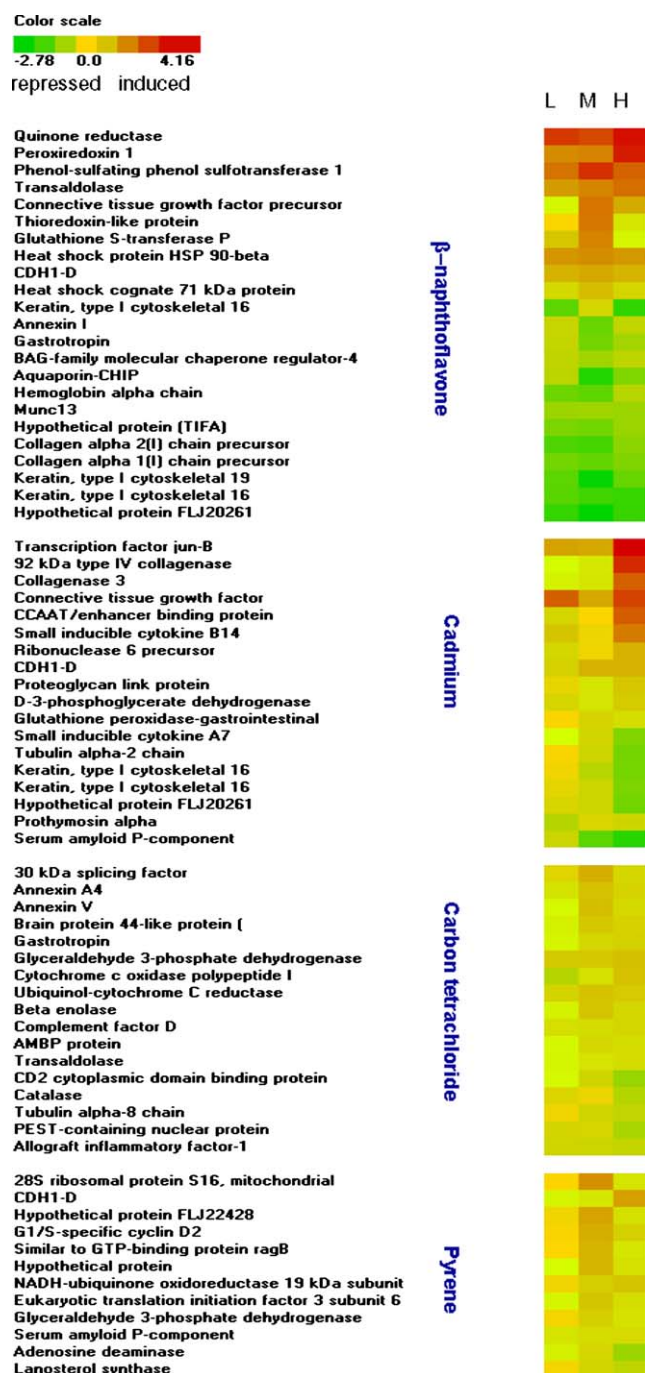


Fig. 2. Examples of genes that showed significantly differential expression in at least 2 sublethal doses of the studied compounds. L, low; M, medium; and H, high doses.

experiments with different compounds pair-wise by average ranks and examples of classes that showed significant difference (Student's *t* test, $p < 0.05$) are shown in Fig. 4. Some of these functional categories include the genes that were discussed above (Fig. 2). For instance, exposure to β -naphthoflavone affected development of central nervous system and transmission of nerve impulse as well as response to xenobiotic stimuli; this chemical

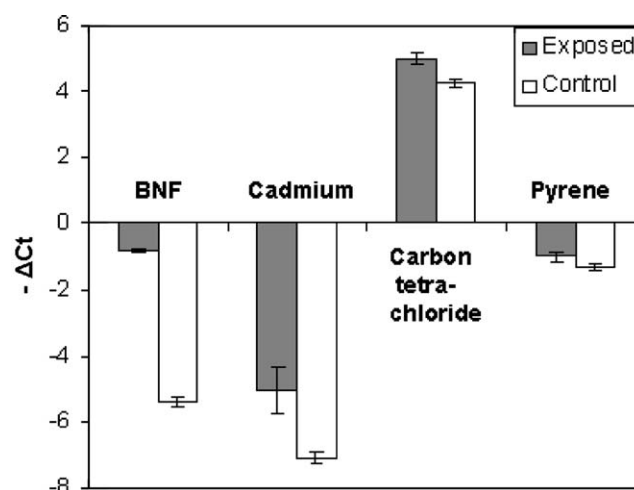


Fig. 3. qPCR, expression of two genes was analyzed for each chemical (Table 1). The negative difference of threshold cycles ($-\Delta C_t$) is proportional to the log ratio of initial copy numbers [20]. All differences between exposed and control fish are significant ($n = 3$, Student's *t* test, $p < 0.05$).

also induced expression of heat shock proteins and other chaperones. Cadmium augmented catabolism of collagen and binding of heparin and glycosaminoglycans, i.e., extracellular matrix being an important target. Response to carbon tetrachloride was best seen in a number of functional classes that are related to the phospholipid structures, lipid metabolism, and glycolysis.

Furthermore, comparison of transcriptomic responses by functional classes indicated some tendencies that could not be seen at the single-gene level. Exposure to β -naphthoflavone induced apoptotic response greater than other compounds, though activation of caspases could also be seen in experiment with cadmium. β -Naphthoflavone enhanced cell proliferation, whereas the negative regulators were suppressed. In contrast, carbon tetrachloride appeared to arrest cell cycle. Response to toxic compounds could involve different mechanisms of signal transduction. For instance, β -naphthoflavone induced the RAS pathway and JAK-STAT cascade, which is associated with cytokine receptors. The latter was also activated with cadmium being inhibited with carbon tetrachloride and pyrene. Protein biosynthesis could be enhanced by β -naphthoflavone and carbon tetrachloride, whereas pyrene decreased expression of genes involved in protein transport. Overall, the studied compounds could affect different pathways of protein degradation. The genes of ubiquitin-dependent protein catabolism were up-regulated with β -naphthoflavone, whereas ubiquitin ligase complex was enhanced with pyrene being suppressed with cadmium and carbon tetrachloride. Proteins binding magnesium and manganese ions were induced, respectively, with carbon tetrachloride and pyrene. Enzymes of several types showed specific response to the studied chemicals.

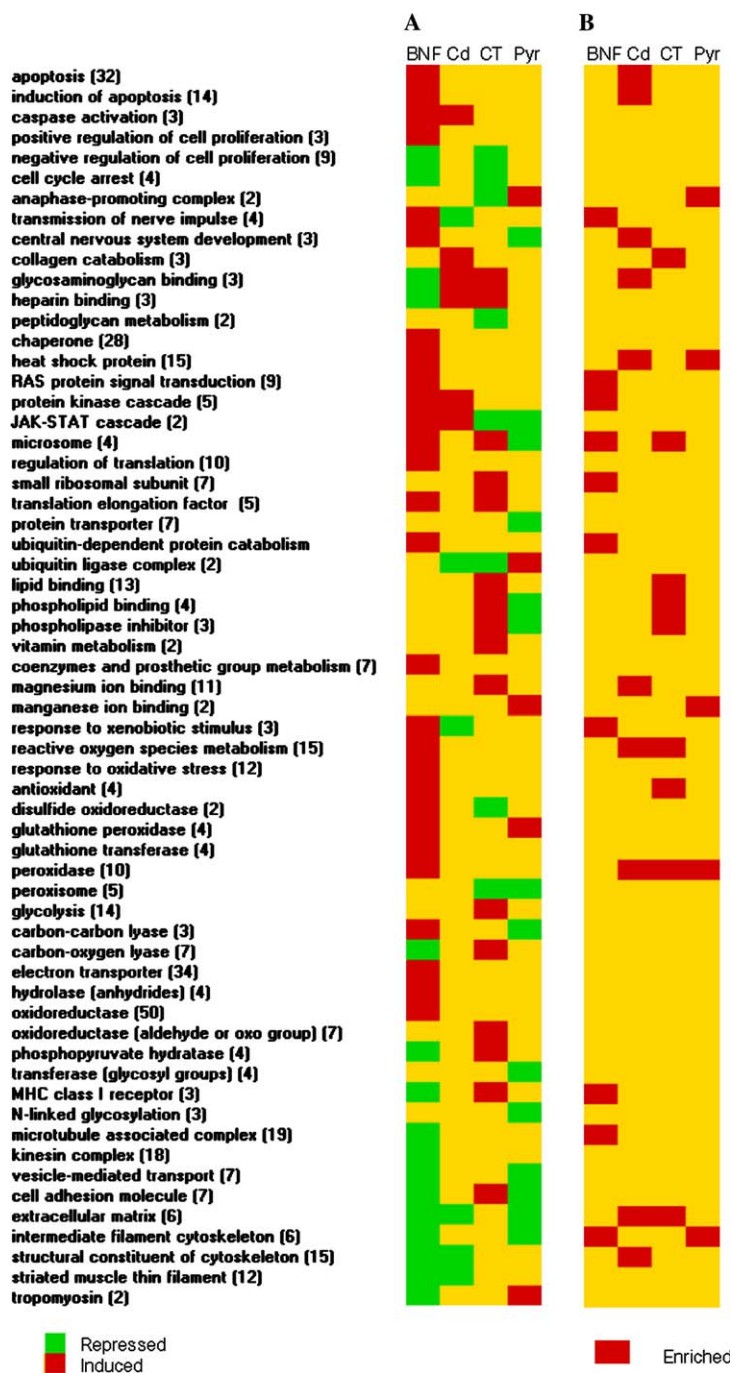


Fig. 4. (A) Functional categories of Gene Ontology that discriminate the toxic effects of β -naphthoflavone (BNF), cadmium (Cd), carbon tetrachloride (CT), and pyrene (Pyr), the numbers of genes in the microarray are given in parentheses. The experiments were compared pairwise by the sums of ranks and the significance was determined with Student's *t* test ($p < 0.05$). (B) Over-presentation of themes in the Medline abstracts.

As expected, exposure of rainbow trout fry to the contaminants affected redox condition and metabolism of oxidative species. β -Naphthoflavone increased expression of antioxidants and a large group of proteins involved with oxidative stress. Glutathione peroxidases were also up-regulated with pyrene, whereas carbon tetrachloride suppressed disulfide oxidoreductase. Carbon tetrachloride and pyrene decreased expression of peroxisome proteins. We also observed marked difference in effects of

contaminants on various structural proteins. β -Naphthoflavone decreased expression of proteins of vesicle-mediated transport system and microtubule associated complex, kinesin complex, tropomyosin, striated muscle thin filament, cytoskeleton, and extracellular matrix; three latter groups were also negatively regulated with cadmium. Pyrene enhanced expression of tropomyosins.

Result of cluster analysis (Fig. 1) revealed that at high doses of contaminants the adaptive reactions could be

masked with general unspecific response. We compared high doses with low and medium doses by the mean ranks and found significant differences (Student's *t* test, $p < 0.05$) in a number of functional classes (Fig. 5). Most of the categories that were enhanced at high doses are related to energy metabolism and mitochondrion. These include proteins of the inner membrane of mitochondrion as well as proteins of electron transport chain, transporters of ions and nucleotides, enzymes of oxidative phosphorylation, cytochrome *c* oxidases, NADH dehydrogenases, and oxidoreductases. Two more groups are transporters of metal ions and proteins involved in translation and protein-disulfide reduction. The genes whose expression decreased at high doses fell mainly into functional classes that are commonly activated in acutely adverse conditions. These are related to stress and immune response, generalized response to chemical substances and abiotic stimuli. Noteworthy, despite suppression of immune system, expression of several cytokines was up-regulated. Exposure to high doses clearly affected receptors, signal transduction, and

a number of metabolic functions (biosynthesis of steroids and lipids and metabolism of nucleotides).

Discussion

This study addresses the potential of newly developed rainbow trout cDNA microarray for environmental toxicology, the main issues being development of biomarkers and elucidation of molecular mechanisms of toxicity. Emphasis was made on comparison of responses to four relatively well-characterized toxic compounds at the sublethal range of doses. By purpose also, we preferred to begin directly with dose-related experiments, i.e., by exposures to low, medium, and high sublethal doses of the model contaminants, to assess the applicability of data for ecotoxicological risk assessment. We asked, whether multiple gene expression profiling will help to develop novel diagnostic markers and add to understanding of molecular mechanisms of toxicity. We used yolk-sac fry, the stage generally considered as the most sensitive one of fish life.

To develop diagnostic markers, we selected a pair of up-regulated and down-regulated genes for each of the studied compounds (Table 1). Cytokeratin and α -tubulin are structural proteins of cytoskeleton. Type IV collagenase is an extracellular zinc-dependent proteinase. Electron transporter thioredoxin participates in various redox reactions. Phospholipase inhibitor annexin (lipocortin) is involved in inflammatory response and cell surface receptor linked signal transduction. Siah 2 protein takes part in GTPase mediated signaling and ubiquitin-dependent protein catabolism. Noteworthy, most of these genes have never been used as markers in ecotoxicology. qPCR confirmed differential expression of these genes.

Basically, microarray analyses provide unique possibility to address many cellular functions, metabolic and regulatory pathways in a single assay. This requires both careful design of microarray as well as development of data mining approaches. We preferred to use a relatively small number of genes and a large part of these was selected by functional categories; every gene was presented with six replicate spots. Dye-swap design of experiment provided robust normalization of gene expression data and high power of statistical analyses (expression of every gene was measured in 12 replicates). This allowed finding of relatively small alterations of expression levels. Random pooling of four individuals in each sample reduced biological variation, however, representing the population average at an exposure level.

Grouping of differentially expressed genes by functional classes is a common way for interpretation of microarray data [16–19] and a number of categorical systems may be used. Gene Ontology [10] is becoming a

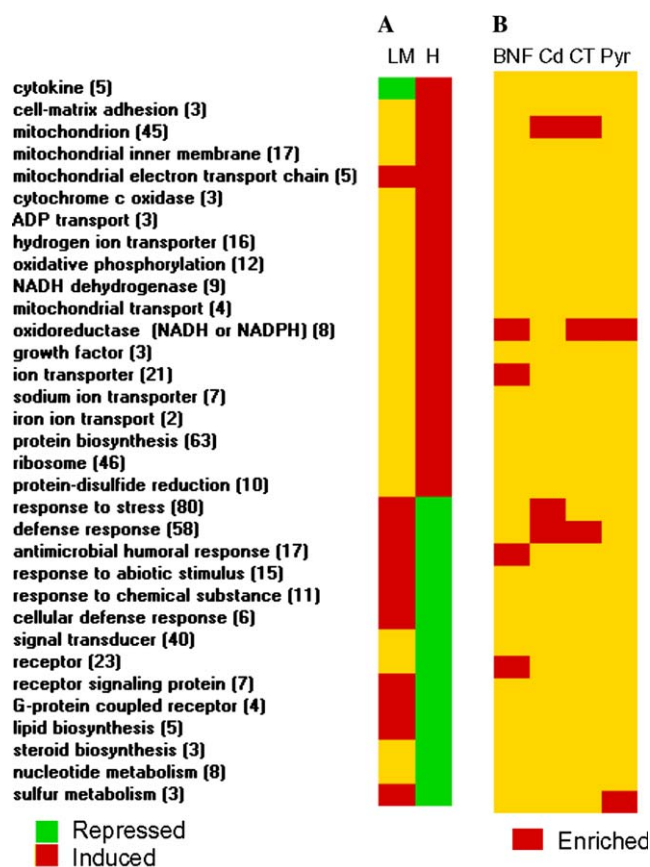


Fig. 5. (A) Unspecific responses to toxicity of four chemicals: functional categories that showed similar tendency in all experiments. High (H) doses were compared with low and medium (LM) doses by the sums of ranks (Student's *t* test, $p < 0.05$); the numbers of genes in the microarray are in parentheses. (B) Over-presentation of themes in the Medline abstracts.

standard for functional classification of genes and, in our hands, this system appeared most informative and useful. Design of experiments made possible to compare contribution of functional categories in transcriptomic response both by chemicals and by doses. Interpretation of these results was enhanced with computer-assisted analysis of Medline abstracts. Relative enrichment of terms in scientific papers indicated molecular mechanisms that were given preference in studies of toxicity of β -naphthoflavone/TCDD, cadmium, carbon tetrachloride, and pyrene. This helped us to indicate the expected and unexpected transcriptomic responses to the studied compounds.

Mining of abstracts predicted preferential activation of heat-shock proteins, RAS pathway of signal transduction, protein biosynthesis, and proteasome protein degradation at exposure to β -naphthoflavone and this was observed in our experiments (Fig. 4). Induction of microsomal proteins by β -naphthoflavone and carbon tetrachloride was also expected. Apoptotic response to β -naphthoflavone was greater than to other compounds, despite stronger association of apoptosis with cadmium, however, the latter induced the genes that activate caspases. Exposure to β -naphthoflavone markedly affected expression of many proteins taking part in the formation of microtubules, kinesin complex cytoskeleton, and extracellular matrix, though mining of abstracts predicted greater impact of other compounds on these structures. The same is true for various aspects of metabolism of reactive oxidative species and responses related to oxidative stress.

Transcription factor Jun B and α -tubulin showed consistent response to cadmium (Fig. 2), being in line with over-presentation of these names in abstracts (Table 2). In the published literature, the observed effects of cadmium on connective tissues and extracellular matrix were also well documented. However, it was difficult to predict powerful enhancement of collagen catabolism, since this theme showed stronger association with carbon tetrachloride. As expected, exposure of rainbow trout to carbon tetrachloride activated genes involved in lipid binding and various aspects of lipid metabolism. Many structural proteins of cytoskeleton, skeletal muscle, and extracellular matrix showed strong response to toxicity, which could be due to active somatic growth of yolk-sac fry.

We also assayed transcriptomic response of rainbow trout larvae to different doses of toxic chemicals. Hierarchical clustering suggested that gene expression profiling could discriminate effects of contaminants at low and medium doses from those at high sublethal levels (Fig. 1). Furthermore, *K*-means clustering found two groups of genes, whose expression increased or decreased with elevation of doses in all four experiments (data not shown). Finally, comparison of low, medium, and high doses revealed mechanisms of general and

unspecific response to toxicity and significant difference was found in a number of closely related functional classes (Fig. 5A). Accordingly, high doses augmented energy metabolism, especially targeting mitochondria, protein synthesis and modification, and transport of metal ions. In parallel, genes that are related to cellular stress, immune, and defense response were down-regulated. Noteworthy, analysis of Medline abstracts showed preferential association of most of these themes with some of the analyzed chemicals (Fig. 5B).

Finally, we must ask whether gene expression studies provide valuable information for risk assessment and environmental protection. Can they serve as a technical source of biomarkers of early warning type or are we dealing merely with an early noise, which is subsequently coped by physiological and metabolic acclimations and compensations? For the time being without definitive chain of causalities even for a single toxicant, we presume that the knowledge on transcriptional expression is a logical addition to more integrative endpoints currently in use more widely. The use of trout cDNA microarray appears, anyway, to be a powerful addition to the repertoire and we currently have to understand ecotoxicity at more integrative levels ranging from tissues to populations.

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